Progress in the use of genetic methods to study insect behavior outside *Drosophila*

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Abstract

In the span of a decade we have seen a rapid progress in the application of genetic tools and genome editing approaches in "non-model" insects. It is now possible to target sensory receptor genes and neurons, explore their functional roles and manipulate behavioral responses in these insects. In this review, we focus on the latest examples from Diptera, Lepidoptera and Hymenoptera of how applications of genetic tools advanced our understanding of diverse behavioral phenomena. We further discuss genetic methods that could be applied to study insect behavior in the future.

Introduction

Insects are the most numerous and diverse animal taxa on the planet. They have evolved different adaptations in sensory function and neural circuitry towards performing basic behavioral tasks such as finding mates, food and oviposition sites [1]. The availability of advanced genetic tools in *Drosophila melanogaster* has allowed us to perform sophisticated genetic experiments to investigate everything from gene expression to brain and behavior. The vinegar fly is a wonderful model for investigating odor- and light-directed locomotion and courtship, but offers little insight into pollination, phyto- or haemotophagy and eusociality. From the perspective of meeting the global challenges of the 21st century *D. melanogaster* fails as it is neither a crop pest nor a disease vector and we are only beginning to understand its natural behaviors [2]. Paradoxically, only limited genetic tools are employed for insects with better studied behavior in their ecological context [3]. The application of genetic techniques in non-drosophilids is often directed by pioneering *D. melanogaster* studies that have uncovered phenotypes for candidate homologous gene targets. However, while a genetic tool can often be successfully ported between species (e.g. fluorescent markers [4], gene editing [5,6] or transgene binary expression systems [7–9]), the behavioral outcome of targeting homologous genes is less predictable and needs to be studied on species-by-species basis [e.g. 10–13].

A number of recent studies have employed gene silencing or editing in Diptera, Lepidoptera, Hymenoptera, Orthoptera, Hemiptera, Coleoptera and Blattodea (**Table 1**, **Box 1**) in order to understand their behaviors. The majority of these studies focused on olfaction, reflecting its importance for insect fitness and survival, and the multi-sensory nature of many natural behaviors. These advances herald exciting times in studying the genetic basis of insect behaviors, with increased focus on the organism itself and reduced focus on its use as a genetic model. Here we review the latest progress in the use of genetic tools in behavioral studies, taking a closer look at insect-plant interaction, social behaviors, human host-seeking and oviposition. We also highlight studies that can potentially help decipher the neuronal basis of behavior.

Box 1. Overview of genetic methods

RNA-interference (RNAi)

The technique of suppressing gene transcription through the application of RNA-interference (RNAi) was first described in 1998 in the nematode *Caenorhabditis elegans* [14]. Since its discovery, this technique has been widely used in insects [15–20] to silence genes of interest by exogenous or endogenous delivery of double-stranded RNA (dsRNA) or small interfering RNA (siRNA). In target cells, dsRNA is cleaved to siRNA by the Dicer enzyme and is incorporated into the RNA-induced silencing complex (RISC) to direct degradation of complementary endogenous mRNA of the targeted gene. The flexibility of this technique is partially attributed to the fact that only the sequence of the gene, and not that of its chromosomal location or regulatory untranslated regions, is required to design dsRNAs. However, the technique is susceptible to variable or no results depending on insect species, gene, tissue, and method of delivery [16,21,22]. Yet, the ability to reduce (knock-down) and not completely ablate (knock-out) the function of a targeted gene at different stages of development permits the analysis of early regulators of sensory behaviors that are also essential for overall survival. Multiple methods of delivery, such as injections at any developmental stage, feeding, transgenic expression and soaking (**Fig. 1**), enable manipulation of insect behaviors in laboratory and the field for research or pest control purposes. Interestingly, pre-blastoderm embryo injections of mRNA has been successfully applied as a forward genetics approach to upregulate gene expression to study sex determination in mosquitoes [23].

Directed mutagenesis by ZFNs, TALENs and CRISPR/Cas9

Reverse genetics is central to associating genes with a biological function. Classic methods of altering genomic DNA using X-rays to induce chromosomal breakage *in situ* revolutionized our ability to associate a particular genomic locus with a behavior [24]. Later, genome sequencing helped map these loci to specific genes and, most recently, gene function was explored through targeted mutagenesis. Targeting a particular gene of interest became feasible for non-model organisms with the help of Zinc-finger nucleases (ZFNs) [25,26] and Transcription activator-like effector nucleases (TALENS) [27]. However, costs and time associated with engineering these proteins prevented a quick adoption of these methods. The discovery of CRISPR/Cas9 system, the part of the bacterial adaptive immune system [5,6,28], permits a fairly quick and inexpensive mechanism for the targeted modification of DNA with the ability to generate deletions from a single base pair to hundreds of kilobase pairs [29]. It is currently the fastest and most effective method of genome editing in diverse organisms from bacteria to human [30]. The application of the technique requires a source of Cas9 protein and the custom-designed guide-RNAs (sgRNAs) that are complimentary to the gene of interest. The sgRNAs bind the Cas9 and deliver it to the desired location in the genome. Cas9 induces a double-stranded break that is naturally repaired either through non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanism. The latter mechanism allows researchers to design specific DNA homology templates surrounding the repair site, adding elements such as transgenes to be incorporated into the target site. In most cases, components of the CRISPR/Cas9 system (Cas9, sgRNAs and a DNA homology template) are injected into a pre-blastoderm embryo. To overcome high costs and workload, and embryo lethality associated with injection, new methods of delivery directly into a gravid female are now being developed [31,32].

Since its introduction as a gene editing tool in 2012, CRISPR/Cas9 system has advanced research in many insect species, including flies [33–38], sandflies [39], mosquitoes [40–43], moths [13,44–47], butterflies [48,49], crickets [50], locusts [51], planthoppers [52], honeybees [53,54], wasps [55], ants [11,12], beetles [56], aphids [57] and psyllid bugs [31].

Transposon Mutagenesis

The workhorse of *Drosophila melanogaster* genetics is the P-element [58]. The P-element is a sequence of nucleotides recognized by a transposase found in wild *Drosophila* and applicable for insertion based mutagenesis in lab strains of *D. melanogaster*. These transposable elements allow researchers to insert genes and gene reporters into the germ line of the vinegar fly driving research in reverse and forward genetics. However, the p-element is narrowly applicable to other insect species. The piggyBac transposable element was discovered in the cabbage looper moth, *Trichoplusia ni* [59,60] and has been applied broadly to generate random insertions of transgenes in non-model insects [61].

Insect-plant interaction

Insects and plants have co-evolved for approximately 400 million years and many insects rely on the sensory perception of plant cues to elicit quick and adaptive behaviors [62]. Plants are also not passive in these interactions, exemplified by the diversity of flower colors or plant odors driven by the selectivity of their pollinators or voraciousness of their pest.

The crepuscular hawkmoth *Manduca sexta* uses both visual and olfactory cues to locate its host plant, the Western Jimsonweed, *Datura wrightii* [3,13], which produces a relatively large, white upright trumpet flower with a strong odor bouquet. Mediating the detection of this floral bouquet are a subset of diversely evolved insect chemosensory receptors, one group of which is encoded by the odorant receptor (OR) genes. The ORs form ligand-gated cation channels with a highly-conserved insect co-receptor ORCO [10,63], and determine the channel's odorant-binding specificity. The ORCO gene is thus necessary for proper function of most olfactory sensory neurons in an insect. Mutating ORCO provides a means of shutting down a large portion of the olfactory system and evaluating its importance in behaviors in insects (**Table 1**). Recently, CRISPR/Cas9 was used to generate an ORCO knock-out (KO) in *M. sexta* [13]. Wind tunnel experiments on ORCO mutants demonstrated that while the nectar-filled and fragrant flower provides a strong visual cue, ORCO-dependent olfaction is needed to complete the sensory behavior of hovering, unfurling the proboscis, and feeding [13]. Interestingly, ORCO-independent sensory processes, such as vision, perception of humidity, and CO_2 do not compensate for the innate behavior involved in seeking out the *Datura* flower (**Fig 2A**). This study also investigated the role of ORCO in hawkmoth plant-seeking for oviposition. The hawkmoth caterpillar is an herbivore and *Datura* is a preferred food source, often to the detriment of the plant. A gravid female hawkmoth evaluates a suitable host plant via olfactory cues from plant leaves [64], and this host-seeking behavior is significantly disrupted in ORCO mutants. However, a number of gravid ORCO-mutant *M. sexta* were still able to locate their host [13], implying that other ORCO-independent olfactory cues may direct this host plant seeking behavior. Thus, the hierarchy of sensory cues and the mode of their integration may vary in multi-sensory behaviors and can only be understood by testing a reverse genetic phenotype in a semi-natural environment. Further implementation of genetic methods, e.g. live imaging of neuronal responses as done in mosquitoes [65,66], could help us understand the gene-specific representation of sensory cues in an insect's brain.

Contact chemoreception mediated by gustatory receptors (GRs) is important for oviposition in many insects, especially in Lepidotpera [67]. Female swallowtail butterflies *Papilio xuthus* evaluate the suitability of a substrate while drumming their front legs against the leaves of their Rutacea (citrus) plant host. Synephrine, a citrus plant alkaloid, induces a physiological response in the female tarsi [68]. Gustatory receptor *PxutGR01* was found to be expressed only in females and respond to synephrine when heterologously expressed in an insect cell line [67]. An injection of dsRNA in the pupae downregulated the PxutGR01 transcript and physiological response to synephrine was reduced in the tarsi of adults. While there was no change in the drumming activity, the oviposition behavior in response to synephrine was reduced in the knock-down individuals, demonstrating that this GR is responsible for the evaluation of synephrine. Laying eggs on the right plant is important since caterpillars need to overcome the plant's defense mechanisms and feed the moment they hatch, and not all leaves provide adequate nutrients to support growth and development. The peripheral sensory system mediating this choice has been studied further in the monophagous silkworm, *Bombyx mori*. This moth is cultivated for its silk cocoons and has been a Lepidopteran model for the development of genetic tools [69]. Additionally, many behaviorally abnormal strains of *B. mori* have been cultivated and their genetic loci characterized. The silkworm feeds exclusively on the leaves of the common mulberry plant and a specific cultivated strain was found to have an abnormal food preference. A putative bitter sensing gustatory receptor *BmorGr66* was identified within the mapped genetic loci of the abnormal strain [70]. The application of CRISPR/Cas9 to mutate the *BmorGr66* led to the silkworm accepting foods like fruits and grains in addition to mulberry leaves [70]. Electrophysiological analyses of the mutants did not reveal any general sweet or bitter contact chemoreception deficit; the ligand for mulberry leaf preference remains to be identified. CRISPR/Cas9 mutation of ORCO in *B.mori* silkworms also determined that OR-related olfaction was important for feeding behaviors; the ORCO-mutants had trouble localizing the mulberry leaves in a test arena [71]. These studies highlight the importance of single sensory receptors for complex phenomena like foraging preference. Identification of genes like *BmorGr66* may further instruct genetic pest control strategies.

Social interactions

Eusocial insects live in complex societies and interact with each other in fascinating ways to maintain social integrity [72,73]. However, understanding the genetic basis of these sensory behaviors has been hindered by the lack of genetic tools, which are particularly difficult to establish when only very few female individuals reproduce sexually, and generation times often span many months. Moreover, these females often have to be isolated to start new colonies. Genetic crossing and outcrossing routines are thus difficult to achieve in eusocial insects in a laboratory setting.

Pheromones play a crucial role in regulating social behaviors in eusocial Hymenoptera like ant communities. Antennal olfactory neurons respond to conspecific cuticular hydrocarbons in C*amponotus floridanus* ants [74], and ORCO-dependent receptors of the ponerine ant *Harpegnathos saltator* respond to its cuticular hydrocarbons and pheromones when ectopically expressed in *Drosophila* [75]. Recent studies have taken the next step in genetic characterization of the role of olfaction for intraspecific communications of two species of ants, using CRISPR/Cas9 for the first time in eusocial insects to mutate the ORCO gene [11,12]. Workers of *Harpegnathos saltator* present a unique advantage that facilitates a genetic modification – all workers of this species normally mate, and can take over the queen's place after the queen dies or is removed from the colony [11]. The unmated workers may thus lay haploid eggs that develop into males, or the workers may be allowed to mate and lay diploid eggs that produce females. Another ant species, the clonal raider ant O*ocer*a*ea biroi* was selected for these experiments because it reproduces asexually via parthenogenesis, thus overcoming the obstacles related to difficult genetic crosses [12]. In addition, these ants are blind, which simplifies analysis of their behaviors in response to multisensory cues.

ORCO mutant individuals of two species showed deficiencies in olfactory response to pheromones and other volatiles, and abnormal social behaviors (**Fig 2B**). For instance, the ORCO-mutant *O. biroi* could not detect and follow the pheromone trail to their nest, spending a significant amount of time wandering. Additionally, a permanent Sharpie marker line drawn on a surface often deters wild type ants from approaching the line, however, the ORCO-mutants were not repelled and often crossed these lines [12]. Both studies also reported a surprising defect in olfactory neurodevelopment. The ORCO-mutants exhibited a dramatic reduction of olfactory receptor neurons in the antennae [12] and the number of glomeruli in their antennal lobe. Interestingly, ORCO mutation leads to no visible changes in the brain of *Drosophila* [10], and only minor reduction in the relative volume of pheromone-specific glomeruli in *M. sexta* [13]. These results reveal that olfactory neurodevelopment in the ant is largely dependent on the presence of functional ORCO, and raise intriguing questions about the role of ORCO and other olfactory genes in neurodevelopment of other insects.

Within the ant colony necrophoric behavior, the removal of dead individuals from the nest by workers, is an important innate behavior that is triggered by olfactory cues from dead individuals [76]. A study on the red fire ant *Solenopsis invicta* showed that a chemosensory protein gene *Si-CSP1*, which is highly expressed in the antenna of workers, is involved in detecting volatile oleic and linoleic acids from dead nestmates and in regulating the necrophoric behavior of *S. invicta* workers. The behavior was suppressed by RNAi through feeding with siRNA mixed into sugar water [77], demonstrating that siRNA feeding is a feasible method of genetic intervention in red fire ants, and could even be a means of population control.

Attempts are currently underway to introduce genetic tools into another eusocial insect, the honeybee. CRISPR/Cas9-mediated genetic editing of the *major royal jelly protein 1* (*MRJP1*) gene and a mushroom-body-specific protein *mKast* were successful (as verified by genotyping) in honeybees but did not affect the normal development of drones [53,54]. These studies pave way for generating genome-edited honeybee workers for investigating their neurodevelopment, innate and learnt behaviors. This work is especially exciting given the economic importance of honeybees as pollinators and the long history of learning and memory studies on honeybees.

Human host-seeking

Insects are vectors of malaria, Zika, Dengue, yellow fever, Chagas and other lethal diseases. Female mosquitoes (**Fig 2C**) and triatomine bugs [78] target their human and animal hosts in order to obtain a blood meal and develop their eggs. Zinc-finger nucleases (ZFNs) were used to mutagenize the ORCO [25] and CO_2 co-receptor

GR3 [26] of *Aedes aegypti* mosquitoes. The mutations impaired the mosquitoes' ability to detect components of human body odor and CO_2, but still left them able to find humans. Mosquitoes with a CRISPR/Cas9-generated mutation for ionotropic olfactory co-receptor, *IR8a*, were impaired in their ability to respond to acidic odorants that are components of human sweat [79]. This mutation also significantly reduced attraction of female mosquitoes to a human arm. The attraction was reduced further, but not abolished, in mosquitoes carrying two mutations, *IR8a+ORCO* or *IR8a+GR3* indicating that other cues outside chemosensation mediate attraction to humans. One of these cues is human body heat. For example, mutation in *Aedes TRPA1* gene affects the mosquitoes' preference for human body temperature (40 °C) and avoidance of warmer objects (50-55 °C) [80]. These studies have highlighted the multisensory and additive nature of sensory cues mosquitoes employ in finding humans (summarized in [81]), drawing certain parallels with plant host-seeking in hawkmoths.

Interestingly, female *Aedes aegypti* mosquitoes discontinue host-seeking for four days after a blood meal, and resume after the eggs have been laid. A recent study has discovered that human neuro-peptide Y (*NPY*) Y2 receptor agonists efficiently target the *Aedes* NPY-like receptor 7 (*NPYLR7*), suppressing mosquito attraction to humans [82]. *NPY* antagonists had the opposite effect, leading to increased host-seeking. Mosquitoes that carried a CRISPR/Cas9-induced mutation in *NPYLR7* resumed host-seeking only one day after the blood meal, in contrast to four days in wild-type mosquitoes. A drug screen, conducted on wild-type and NPYLR7-mutant mosquitoes, identified six NPYLR7-specific agonists that suppress mosquito attraction to humans. These findings suggest an exciting new pathway for behavioral analysis of mosquitoes and the potential for vector disease control by deploying mosquito drug feeders.

The neuronal circuits that underlie mosquito host-seeking are currently unknown. Thus the next step is to investigate how multimodal sensory stimuli and systemic signals are processed in the mosquito brain. First steps in this direction have been taken in *Anopheles gambiae* [83,84] and *Aedes aegypti* [85] by employing the fluorescent calcium indicator GCaMP to image live neuronal responses from the peripheral organs and the brain of mosquitoes. The same approach has been taken to study *Aedes* oviposition choices [65,66].

Oviposition

Drosophila neuroscience heavily relies on transgenic lines, and in particular on three orthogonal binary expression systems (reviewed in [86]). Reporter transgenes are especially useful for labelling neurons, monitoring or manipulating their functional responses. Generating transgenic lines in other insects has, until recently, been hampered by the need to identify and clone out the native enhancer and promoter region for the gene of interest (although see [83,85,87,88]). The advance of CRISPR/Cas9 has removed this requirement, and now allows us to introduce a transgene, with a T2A or a similar linker, immediately into or after the coding sequence of a gene [89]. By using live Ca^{2+} imaging of genetically encoded activity indicators, we can now investigate the neuronal basis of the observed behavioral phenotypes. This method has been elegantly used to study oviposition choices in *Aedes aegypti* [65,66] (**Fig 2D**). Gravid mosquito females lay their eggs in or near water sources, because their larvae and pupae are aquatic. Female *Aedes*, mutant for the *pickpocket* cation channel subunit gene *ppk30*, lay fewer eggs and fail to avoid water with high salinity that is harmful for their larvae [66]. Live Ca^{2+} imaging of ventral nerve cord that is innervated by ppk301 expressing neurons from the mosquito legs, has shown that these neurons responds both to water and to NaCl, implying that there must be a parallel neuronal pathway that prevents oviposition in salty water in wild-type *Aedes*. Live Ca^{2+} imaging from the *Aedes* antennal lobe has been used to observe sparse neuronal responses to geosmin, an oviposition attractant [65]. The preference for geosmin has been abolished in ORCO mutant mosquitoes, indicating that, as in *Drosophila*, geosmin binds an ORCO-dependent receptor. *Drosophila*, however, find geosmin repulsive [90] and avoid it in oviposition and other assays.

In summary, these latest studies have shed light on general principles that guide insect behavior. Not surprisingly, complex behaviors such as host-seeking and oviposition in moth and mosquitoes are controlled by multisensory cues. The relative importance of these cues is different for different behaviors, and depends on the internal state of the animal (fed, hungry, host-seeking, etc). Mutations of the highly conserved ORCO gene in different species lead to strikingly different developmental and behavioral consequences, highlighting the necessity of

an era of comparative genetic studies. These are also instrumental for the development of pest and disease vector control strategies.

Outlook

CRISPR/Cas9 provides a unique opportunity to use gene editing to study the molecular and neuronal basis of insect behavior, ranging from sensory perception to memory formation and retrieval [50,91]. Either mutating a gene of interest or simultaneously introducing transgenes into precisely defined locations with CRISPR/Cas9 would permit the functional re-programming of neurons. The successful use of transgenes to monitor neuronal responses in mosquitoes will be undoubtedly followed by similar studies in other non-model insect organisms. Work on *Drosophila* has developed multiple methods for activating or silencing neurons by ectopic expression of sensory receptors or ion channels [86,92]. These techniques are now being adapted to other insects [e.g. 93], promising us greater understanding of the neural basis of insect behaviors.

Gene knockouts deliver a unique opportunity for observing the comparative evolution of gene function. For example, the ORCO gene knock out has been generated in 8 species (**Table 1**). ORCO is a highly conserved gene with putative chaperone function and forms functional co-receptors with the highly diverse ORs. OR gene numbers range from 10 to > 300 across species and are tuned to diverse natural ligands [1,94]. The Orco KO has consistently demonstrated disrupted neurophysiological responses to a range of odorants and pheromones. However, insect OR-mediated behaviors distinctively integrate with other sensory modalities (**Fig 2**). For instance, copulation behaviors continue to occur in ORCO KO *D. melanogaster*, presumably through the flexible multi-sensory nature of their mating cues. On the other hand, the strict OR-mediated perception of pheromones is critical for copulation behaviors in some Lepidoptera [13,*47*]. A more striking example involves the role of ORCO in neurodevelopment, where the loss of ORCO leads to dramatic reduction and loss of olfactory glomeruli and olfactory sensory projections from the antennae, indicating a developmental role for ORCO via an unknown mechanism in ants [11,12]. The application of genetic techniques to other genes and their respective homologues will no doubt advance our understanding of many novel biological phenomena based on expanding comparative observations.

However, care should be taken in understanding certain unforeseen effects of current gene editing techniques. CRISPR-Cas9 may introduce unintended mutations beyond the targeted genes and *in silic*o methods of off-target detection are often unverified in current non-model organisms. These off-target effects may provide misleading information for behavioral phenotypes or disrupt other factors involved in fitness or fecundity. Especially when a genetic rescue lines are not feasible, techniques in testing off-target effects *in vivo* should be considered. Research is quickly advancing in the development of rapid and accurate techniques applying methods in next generation sequencing to identify sites that go through the natural cellular nucleotide repair mechanism after CRISPR application, providing a reliable and un-biased method of off-target detection in any organism [95,96].

Advances in genetic techniques in other insect species will also have practical implications in pest management of major crop and disease vectors. For example, new methods of RNAi delivery now allow applications of it in field conditions for crop protection [17,19]. Additionally, a combination of CRISPR/Cas9 and RNAi can lead to the generation of more insects susceptible to RNAi transcript downregulation. Releases of sterile [97] or bacteria-carrying [98] mosquitoes have been adopted as methods to limit mosquito population. Gene-drive technology now allows us to propagate various genetic modifications and transgenes throughout an insect population [41]. These modifications do not need to eliminate an insect population, but may also rely on manipulating insect behavior, e.g. to divert them from economically important crops or from ourselves. Ultimately the application of these techniques and the observations gained from different insects may provide the conceptual framework to better address these challenges.

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Figures

Figure 1. **Ways to deliver RNAi in insects.**

A. Soaking. Immersion in dsRNA solution against a detoxification enzyme gene has been successfully used in adult fleas [99]. The fleas were incubated at 4C, which excludes active ingestion of dsRNAs.

B. Feeding. RNAi feeding has been applied in e.g. larval mosquitoes [100–103] and *Tribolium* [104], triatomine bug nymphs [105], caterpillars [106], ants [76] and aphids [104,107]. dsRNA may be mixed directly into food [76,104], or presented in the form of nanoparticles [100,101] that slow down the degradation of dsRNA. Bacteria [102] and yeast [103] have been genetically engineered to produce siRNA. Finally, plants may be genetically modified to produce siRNA or sprayed with dsRNA against insect genes [107].

C. Injections. Injection of dsRNA or siRNA is the most common laboratory delivery method. Injections may be given at any of an insect's life stages (e.g. embryos [23,108], larvae [109–111], pupae [91,112–114], adults [115,116]). While labor-intensive, this method normally provides the highest efficiency of gene silencing, with the caveat that giving the injection may impair an animal's survival.

D. Transgenes. Transgenic expression of dsRNA is most commonly used in *Drosophila*, where thousands of UAS-RNAi lines have been established, and may now be used by simple genetic crosses with a driver line of interest. The same transgenic approach is feasible in other insects, but the need to create a stable transgenic line has so far prevented its implementation.

Figure 2. **Insect behaviors, studied with genetic tools.**

A. Insect-plant interactions. Flowers provide visual and olfactory cues (odor bouquet, relative humidity and CO_2), while the leaves of the plant also provide olfactory and gustatory cues for adult female butterflies and moths, and their caterpillars. ORCO-mutant *Manduca sexta* moths are impaired in their foraging behaviors [13] (left). Caterpillars choose their food based on its taste. *Bombyx mori* caterpillars with mutated GR66 receptor expanded their food preference from mulberry leaves to fruit and grains [70] (right).

B. Social behaviors. Social behaviors of ants heavily rely on olfactory perception of pheromones. Recent studies have shown that ORCO-mutant ants are seriously impaired in their social interactions, indicating that ORCO-dependent olfactory receptor neurons are necessary for pheromone perception [11,12].

C. Human host-seeking. Female mosquitoes, as moths, integrate multiple sensory cues to find their human host. Mutations in ORCO [25], GR3 [26] and IR8a [79] receptors that detect human body odors, CO_2 and acidic components of human sweat respectively, have significantly reduced the ability of *Aedes aegypti* to find humans.

D. Oviposition. Female mosquitoes lay their eggs in or near water, and their larvae and pupae develop in water. Thus, oviposition sites need to be carefully selected by the females. Two recent studies have found that *Aedes aegypti* mosquitoes prefer to lay their eggs in geosmin-scented water [65], and tend to avoid salty water [66]. Neurons that respond to salt and water were found in the mosquitoes' legs, and geosmin-sensing neurons – in the antennae.

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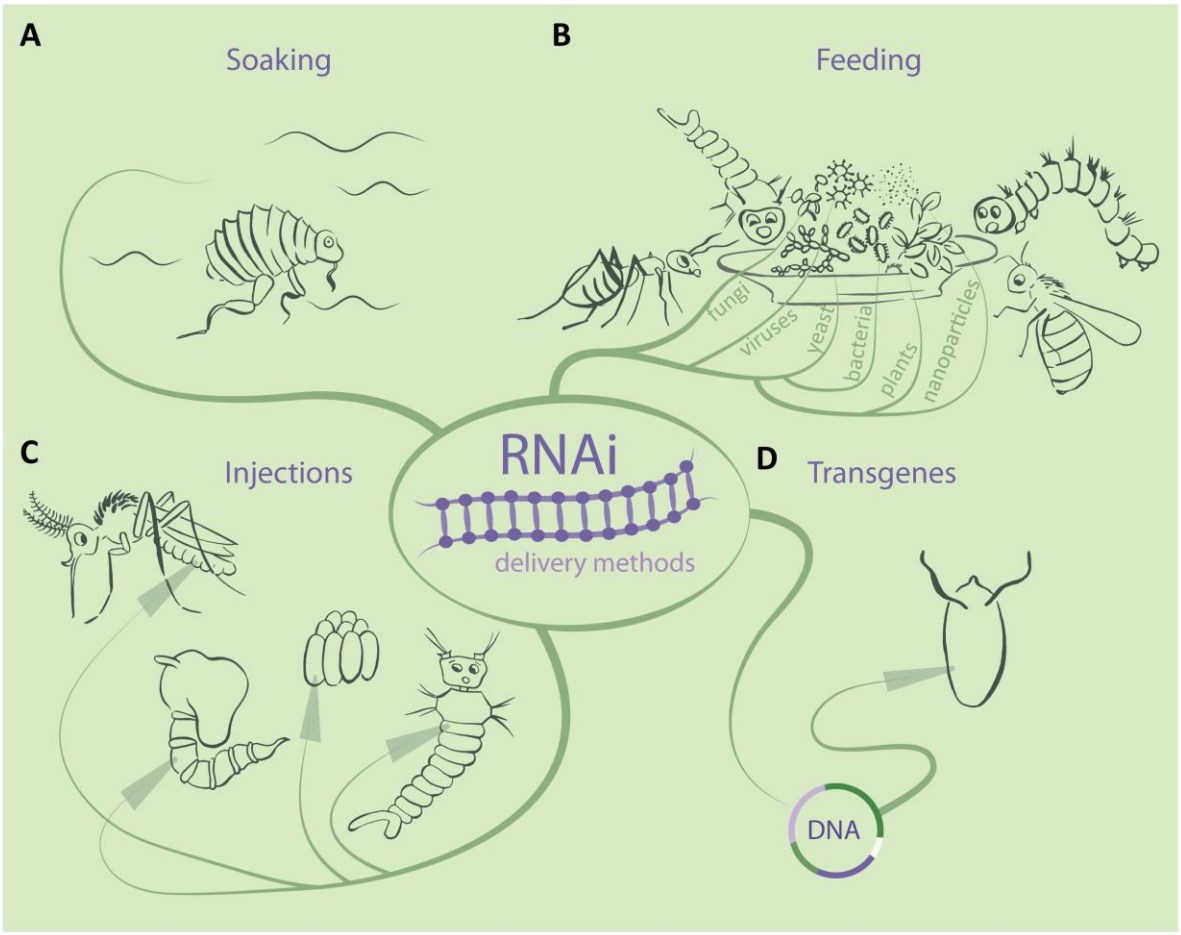
Table 1. Applications of genetic methods to study behavior (non-exhaustive list)

Order	Species	Method	Target	Phenotype	Reference
Diptera	*Aedes aegypti*	chitosan-siRNA nanoparticle feeding	*SEMA1a*	Impaired larval light avoidance due to improper targeting of photoreceptor neurons	[100]
	Aedes aegypti	chitosan-siRNA nanoparticle feeding	*SEMA1a*	Impaired larval yeast attraction due to improper targeting of olfactory neurons	[101]
	Aedes aegypti	CRISPR/Cas9; RNAi injection	*DOP1*	Impaired olfactory learning	[91]
	Aedes aegypti	ZFN	*ORCO*	Loss of OR-mediated olfaction. Disrupted host localization	[25]
	Aedes aegypti	ZFN	*GR3*	Impaired CO_2 detection and host localization	[26]
	Aedes aegypti	ZFN	*TRPA1*	Impaired avoidance of high temperatures	[80]
	Aedes aegypti	CRISPR/Cas9	*PPK301 (also 304, 216, 306)*	Impaired oviposition decisions in response to salty water	[66]
	Aedes aegypti	CRISPR/Cas9	*NPYLR7*	Abnormal host-seeking after a recent blood meal	[82]
	Aedes aegypti	CRISPR/Cas9	*IR8a*	Impaired detection of lactic acid and host localization	[79]
	Aedes aegypti	GCaMP imaging	*ORCO, Ubi-GCamp6s*	Olfactory responses to geosmin observed *in vivo*. Demonstration that geosmin is oviposition attractant.	[65]
	Aedes aegypti	GCaMP imaging	*Ubi-GCamp6s*	In vivo recordings from antennal and optic lobes, evidence of visual - olfactory integration.	[117]
	Anopheles gambiae	RNAi injection	*OR7, OR40, IR76b*	Impaired larval olfactory behavior	[109]
	Anopheles gambiae	RNAi injection	*TRPA1*	Impaired larval thermotaxis	[110]
	Culex quinquefasciatus	RNAi injection	*OR37, OR99*	Impaired oviposition preference for 4-ethylphenol	[113]
Lepidoptera	*Spodoptera littoralis*	CRISPR/Cas9	*ORCO*	Disrupted antennal function towards plant host and pheromone volatiles. Disrupted mating.	[47]
	Spodoptera exigua	RNAi injection	*Se-uv, Se-bl, Se-lw*	Phototaxis towards green light	[118]
	Manduca sexta	CRISPR/Cas9	*ORCO*	Disrupted plant host localization and foraging behaviors. Disrupted mating	[13]
	Ostrinia furnacalis	TALEN	*ORCO*	Ablated pheromone response	[119]
	Bombyx mori	CRISPR/Cas9	*GR66*	Feeding assay used to determine a gustatory receptor involved in the deterring generalist feeding behavior	[70]
	Bombyx mori	CRISPR/Cas9	*ORCO*	Pheromone detection	[71]
	Papilio xuthus	RNAi	*PxutGr01*	Tarsal contact chemosensation of plant host compounds	[68]

	Helicoverpa armigera	RNAi injection	Sex peptide receptor	Oviposition and ovary development	[120]
	Danaus plexipus	ZFN, TALENs, CRISPR/Cas9	*CRY2, CLK*	Group eclosion behavior	[49,121]
	Heliconius melpomene, Heliconius cydno	QTL analysis		Mating preference	[122]
Hymenoptera	*Ooceraea biroi*	CRISPR/Cas9	*ORCO*	Deficiencies in social behavior and fitness. Disrupted antennal lobe development	[12]
	Harpegnathos saltator	CRISPR/Cas9	*ORCO*	Deficiencies in social behavior and fitness. Disrupted antennal lobe development	[11]
	Solenopsis invicta	RNAi feeding	*Si-CSP1*	Chemosensory protein, involved in necrophoric behavior	[76]
	Nasonia vitripennis	Genetic crosses, RNAi, hybrids, QTL analysis	*NV10127-29*	Production and perception of male sex pheromone components	[123]
	Nasonia vitripennis	Hybrids, genotyping		Egg-laying preference	[124]
Orthoptera	*Locusta migratoria*	CRISPR/Cas9	*ORCO*	Olfactory response to conspecifics	[51]
	Locusta migratoria	RNAi injection	*CSP3, TO1*	Olfactory response to conspecifics	[125]
	Gryllus bimaculatus	CRISPR/Cas9	*DOP1*	Appetitive and aversive olfactory learning	[50]
	Gryllus bimaculatus	RNAi injection	*OA1, DOP1, DOP2*	Appetitive and aversive learning	[126]
Coleoptera	*Tribolium castaneum*	RNAi injection	TRP channels	Motor behaviors based on anatomical defects of hind leg folding; tonic immobilization	[127]
	Tribolium castaneum	RNAi injection	*TcTβH*	Mobility	[128]
	Tribolium castaneum	RNAi injection	*TRPA1*	Thermotaxis	[129]
	Tenebrio molitor	RNAi injection	*ORCO*	Impaired mate recognition	[130]
Hemiptera	*Rhodnius prolixus*	RNAi injection	*ORCO*	Impaired host localization, ecdysis, survival, oviposition rate and blood ingestion	[78]
	Nilaparvata lugens	RNAi injection	*CSP8*	Decreased olfactory attraction	[131]
	Laodelphax striatellus	RNAi feeding	*ORCO*	Olfactory host-seeking	[132]
Blattodea	*Periplaneta americana, Blattella germanica*	RNAi injection	*CRY1, CRY2, TIMELESS*	Responses to magnetic field	[133]
	Periplaneta americana	RNAi injection	*Opsins Trp Channels*	Electrophysiological characterization of phototransduction	[134]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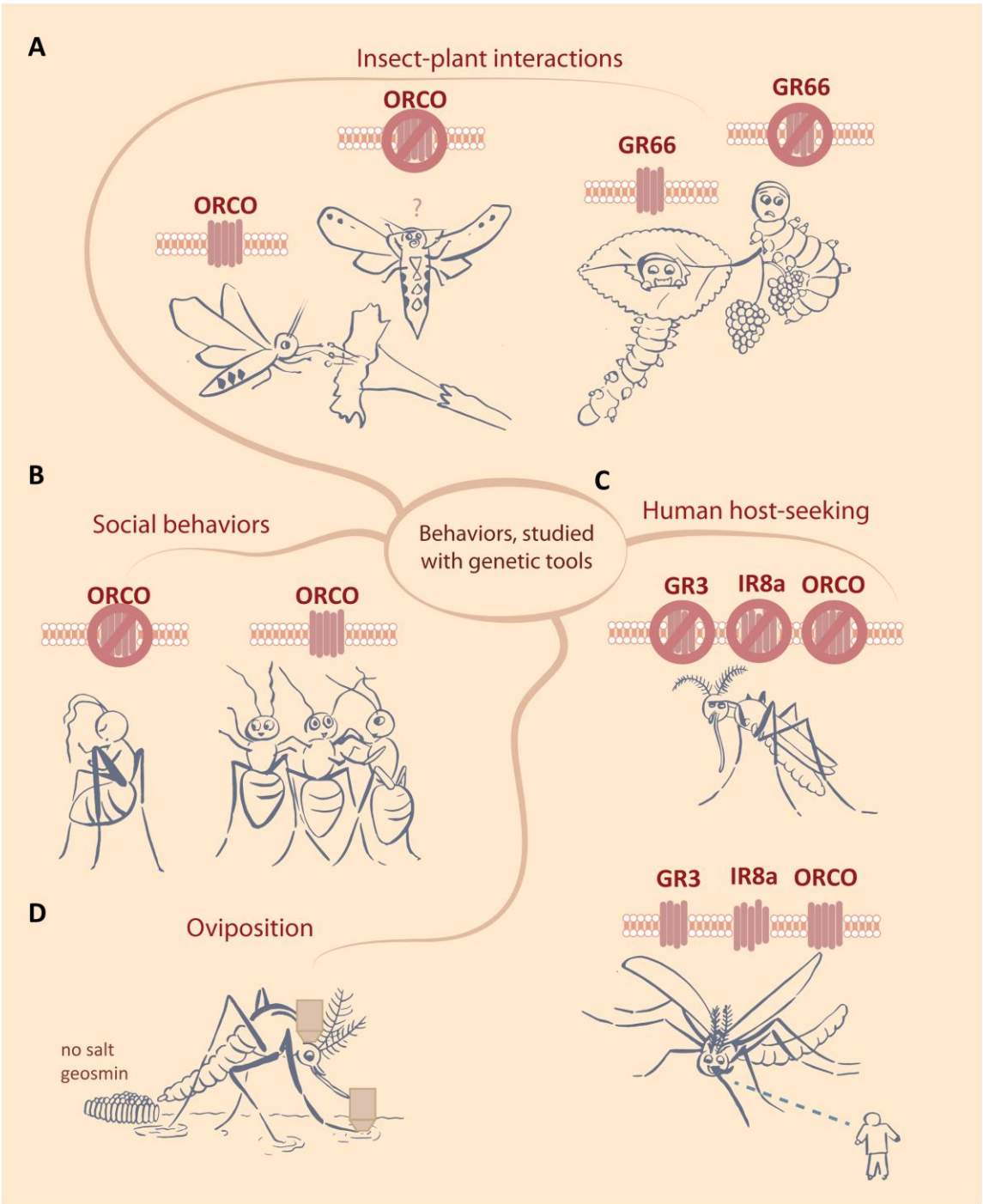
A
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no salt
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